Tumorigenic activity of the BCR–ABL oncogenes is mediated by BCL2

(apoptosis/interleukin 3/leukemia/Philadelphia chromosome/tumor development)

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ABSTRACT  BCR–ABL is a chimeric oncogene generated by translocation of sequences from the c-abl protein-tyrosine kinase gene on chromosome 9 into the BCR gene on chromosome 22. Alternative chimeric proteins, p210BCR–ABL and p190BCR–ABL, are produced that are characteristic of chronic myelogenous leukemia and acute lymphoblastic leukemia, respectively. Their role in the etiology of human leukemia remains to be defined. Transformed murine hematopoietic cells can be used as a model of BCR–ABL function since these cells can be made growth factor independent and tumorigenic by the action of the BCR–ABL oncogene. We show that the BCR–ABL oncogene can cause apoptotic death in these cells by inducing a Bcl-2 expression pathway. Furthermore, BCR–ABL-expressing cells revert to factor dependence and nonmalignant after Bcl-2 expression is suppressed. These results help to explain the ability of BCR–ABL oncogenes to synergize with c-myc in cell transformation.

Hematopoietic growth factors are required to support the proliferation, survival, and differentiation of progenitor cells (1–3). Leukemic conversion of these cells to an autonomous growth state implies that specific genes must be activated to uncouple cell proliferation/differentiation control and to generate intracellular signals that can substitute for growth factor requirements. A well-characterized example in the hematopoietic system involves the rearrangements of the BCR and ABL genes in Philadelphia chromosome-positive (Ph¹+) chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (4–13). Depending on the precise breakpoint within the BCR gene, fusion proteins of 210 kDa (p210) or 190 kDa (p190) are produced (4, 6, 7, 9, 14–16). p210 and p190 BCR–ABL oncogenes contain identical ABL-derived sequences, respectively, but differ in the number of BCR-encoded amino acid residues. The tyrosine kinase activity of the BCR–ABL proteins strongly correlates with their transforming potential in tissue culture and is higher for the 190-kDa form, which is characteristically associated with acute leukemias (12, 14, 17).

Analysis of the mechanism of oncogene action can be studied in cell lines—often in NIH 3T3 fibroblasts. The latter are not transformed by either of the two BCR–ABL oncogenes or by v-abl itself (18). However, it has been shown that BCR–ABL[170] and v-abl convert the factor-dependent Ba/F3 cell line into factor independence and make it tumorigenic (19), but the mechanisms whereby this happens are unknown. Understanding these mechanisms requires identification of the downstream proteins that affect transcriptional control or cell survival. MYC is a candidate gene whose transcription is activated by some tyrosine kinase oncogenes as well as by growth factor stimulation (20, 21). Recent data have shown that MYC is essential for BCR–ABL transformation (22). However, an increase in MYC protein cannot be solely responsible for the transforming effect of BCR–ABL oncogenes, because MYC overexpression does not substitute for the BCR–ABL oncogene transformation phenotype. Therefore, other components in addition to MYC must be required to reconstitute the BCR–ABL oncogene transformation signal.

In the present study, we have examined the role of BCR–ABL oncogenes in the context of hematopoietic cell transformation using as a model system the Ba/F3 cell line (23). We investigated the possibility that BCR–ABL expression may function in Ba/F3 cells by inhibiting apoptosis and consequently prolonging cell survival. We show that the BCR–ABL oncogenes prevent apoptotic death in these cells by inducing a Bcl-2 expression pathway. We further show that Ba/F3 cells expressing BCR–ABL revert to factor dependence and nonmalignancy after Bcl-2 expression is suppressed. These findings implicate the activation of Bcl-2 function as an important component in BCR–ABL-mediated transformation and can also explain the ability of BCR–ABL oncogenes to synergize with MYC in cell transformation.

MATERIALS AND METHODS

Cell Culture. Cell lines used include Ba/F3 (23), K562 (24), and DoHH2 (25). Ba/F3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10% WEHI-3B-conditioned medium as a source of interleukin 3 (IL-3). DoHH2 and K562 cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS and 5 × 10⁻⁵ M 2-mercaptoethanol.

Plasmid Construction. The pAbc12 plasmid contains the mouse Bcl-2 cDNA in antisense orientation and the PGK-Hyg cassette into the pEF-BOS expression vector (26). The final product was confirmed by DNA sequence analysis.

Cell Transfection. Ba/F3 cells were transfected by electroporation with 25 μg of KW3 expression plasmid and 25 μg of E1A2 plasmid along with 2 μg of MC1-neo expression vector. Cell lines were analyzed by Northern blotting for BCR–ABL expression and demonstrated resistance to IL-3 withdrawal. BCR–ABL-expressing Ba/F3 cells were transfected by electroporation (960 μF, 220 V) with 25 μg of the pAbc12 vector. Hygromycin-resistant cells were selected by using 600 μg/ml of hygromycin B (Calbiochem) for 14 days. Clones were screened for high-level Bcl-2 expression by Northern blotting. The antisense Bcl-2 oligonucleotide used as a probe comprises the first 34 bases of the coding sequence of mouse Bcl-2 cDNA.

RNA Analysis. Total cytoplasmic RNA (10 μg) was glyoxylated and fractionated in 1.4% agarose gels in 10 mM Na₂HPO₄ buffer (pH 7.0). After electrophoresis, the gel was blotted onto

Abbreviations: CML, chronic myelogenous leukemia; FL, follicular lymphomas; IL-3, interleukin 3.

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Hybond-N (Amersham), UV-cross-linked, and hybridized to $^{32}$P-labeled probes.

**DNA Analysis.** Low molecular weight DNA was isolated as follows. Cells were collected into 1.5 ml of culture medium and microcentrifuged for 5 min at 13,200 rpm (3000 × g), and the pellet was suspended in 300 µl of protease K buffer. After overnight incubation at 55°C, DNA was ethanol-precipitated, suspended in 200 µl of TE buffer containing 50 µg·ml$^{-1}$ of RNase A, and incubated at 37°C for 2 h. DNA was extracted with phenol and chloroform and precipitated with ethanol. Aliquots of DNA (2 µg) were electrophoresed on 2% agarose gels, which were stained with ethidium bromide and photographed.

**Tumorigenicity Assay.** To test the tumorigenicity of the various cell lines, 4- to 6-week-old athymic (nude) male mice were injected subcutaneously on both flanks with 10$^6$ cells resuspended in 200 µl of phosphate-buffered saline (PBS). The animals were examined for tumor formation for up to 2 months.

**RESULTS**

**BCR-ABL Oncogenes Inhibit Apoptosis of Ba/F3 Cell Line After IL-3 Withdrawal.** Expression plasmids encoding BCR–ABL$^{p210}$ and BCR–ABL$^{p210}$ proteins were introduced into the Ba/F3 cell line. Stable lines were established and BCR–ABL expression was analyzed by Northern hybridization analysis (Fig. 1A). BCR–ABL transforms the IL-3-dependent Ba/F3 cell line to factor independence and tumorigenicity (19) (Fig. 1B; Table 1). The effects of BCR–ABL expression on cell growth were evaluated by analyzing internucleosomal DNA cleavage leading to the formation of DNA ladders in agarose gels, which is a hallmark of apoptosis. A DNA cleavage ladder was observed in the Ba/F3 cells undergoing apoptosis in response to IL-3 withdrawal but not in BCR–ABL-expressing Ba/F3 cell lines (Fig. 1C). Moreover, transfection of the human BCR–ABL oncogenes into the Ba/F3 cell line allowed these cells to survive in the absence of IL-3 not only under optimal (10% FCS) culture conditions but in serum-deprived conditions as well (Fig 1D). These experiments imply that BCR-ABL oncogenes, like other transforming genes, protect against programmed cell death rather than, for example, inducing the autocrine expression of a growth factor (19). These results, together with recent data (27, 28), can explain the massive accumulation of cells in the Ph$^1$ human leukemias, where the leukemic progenitors display normal rates of cell proliferation (27–29).

**Expression of Bcl-2 in BCR-ABL-Expressing Ba/F3 Cells.** The clinical syndromes produced by BCR-ABL$^{p210}$ (CML) and by BCL-2 (follicular lymphoma; FL) are similar, in that both CML and FL exhibit an indolent phase that leads to the development of aggressive malignant phenotypes. Moreover, hematopoietic cell lines transfected with BCL-2 show a relative resistance to the apoptotic death that normally follows growth factor withdrawal (30). Therefore, BCR–ABL oncogenes may suppress apoptosis in a manner similar to that seen for the BCL2 gene in FL (31). We thus examined the possibility that the effect of BCR–ABL oncogenes in cell survival could be BCL2-mediated. Studies at the mRNA level were performed and results of Northern blot hybridizations are shown in Fig. 2. The human BCL2 probe detected a major mRNA transcript in both the p190 and p210 BCR–ABL-expressing Ba/F3 cell lines similar to the size of that found in the DoHH2 cell line, which contains the typical t(14;18) translocation associated with FL (25). The presence of the mouse Bcl-2 protein was demonstrated by Western blot analysis (data not shown). This also compares to the Ph$^{1}$-K562 cell line (24), which expresses the normal-sized BCL2 transcript of 7.5 kb (Fig 2A) and has the BCL2 protein (32).

**BCR-ABL Oncogenes Protect Cells from Oxidative Damage.** Apoptosis can be induced in cell cultures by various treatments and if the effect of BCR–ABL oncogenes in apoptosis is BCL2 mediated, protection of cells by BCR–ABL from oxidative damage will be similar to a characteristic BCL2 effect discovered recently (33). Stable transfectants of Ba/F3 cells bearing BCR–ABL were exposed to exogenous hydrogen peroxidase, which kills cells in a dose-dependent manner (Fig. 3). BCR–ABL-expressing Ba/F3 cells were completely protected from the lethal effects of 0.25–0.5 mM hydrogen peroxidase (Fig. 3 A and B). A dose of 1.0 mM resulted in a more rapid cell killing and showed no survival advantage with BCR–ABL (Fig. 3C).

**Suppression of Bcl-2 Expression Blocks Transformation by BCR-ABL Oncogenes.** The above results provide evidence for the idea that BCR–ABL expression protects cells from apoptosis by inducing a Bcl-2 pathway. In that case, some modi-
Table 1. Growth properties of BCR-ABL-expressing Ba/F3 cells

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<th>Cells</th>
<th>Growth in selective medium</th>
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<tr>
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<td>Ba/F3</td>
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<td>Ba/F3+p120</td>
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DISCUSSION

Bcl-2 is an Important Component in BCR-ABL-Mediated Transformation. The biological activities of the BCR-ABL oncogenes were unraveled by analyzing the effects of BCR-ABL expression on pro-B murine Ba/F3 cell growth. Ba/F3 cells ceased to proliferate and progressively died after IL-3 deprivation by a process of nuclear fragmentation characteristic of apoptosis. We have thus shown that, in the same experimental conditions, BCR-ABL expression protects Ba/F3 cells from activating their intrinsic suicide program, a situation that recapitulates the effects of other transforming proteins on survival (30, 31, 33). This biological activity of the BCR-ABL oncogenes in the Ba/F3 cell lines recapitulates a critical feature of the Ph1 + leukemia: accumulation of leukemic blasts with low intrinsic proliferation activity (27-29).

Our further findings define the molecular basis of BCR-ABL modulation of cell survival. Data presented here show that Bcl-2, which is not normally expressed in Ba/F3 cells, is activated by expression of BCR-ABL oncogenes and mediates the prevention of apoptosis in the Ba/F3 system. Moreover, modification of the growth and tumorigenicity of these BCR-ABL-expressing Ba/F3 cells occurs by altering the level of Bcl-2 expression. Our findings are consistent with a model in which Bcl-2 is downstream in the transformation signal pathway of BCR-ABL oncogenes. As further evidence of this, Bcl-2 expression is significantly reduced in these cells when expression of BCR-ABL oncogenes is inhibited both by using antisense RNA and also by transfecting with a zinc finger peptide, which blocks BCR-ABL transcription (34) (data not shown).

However, an increase in Bcl-2 expression cannot be the only event involved in transformation by BCR-ABL oncogenes. The reasons are as follows. First, although BCL2 plays an important role in tumorigenesis of t(14;18)-bearing lymphomas (e.g., in follicular lymphoma), deregulation of BCL2 may not be oncogenic by itself (35). Second, constitutive BCL2 expression does not result in growth factor-independent lines and therefore replaces not the growth signals induced via growth factor receptors but only the survival signals (36). Thus, other components in addition to Bcl-2 are required to reconstitute the BCR-ABL oncogene transformation signal. In this connection, the ability of BCR-ABL oncogenes to transform cells appears to be dependent on the function of MYC, although MYC overexpression does not substitute for the BCR-ABL oncogene transformation phenotype (22). Moreover, recent

Fig. 2. Bcl-2 expression in BCR-ABL-expressing cells. Normal-sized Bcl-2 transcript of 7.5 kb is present in Ba/F3+p190 (lane 2), Ba/F3+p210 (lane 3), DoHH2-2 (lane 4), and K562 (lane 5) cell lines. Cellular RNA was hybridized to a human BCL2 cDNA probe (A), to an ABL probe (B), and to a mouse \( \beta \)-actin cDNA (C). Autoradiography was for 24 h at –70°C.
Fig. 3. Viability of transfected cells during hydrogen peroxidase-induced apoptotic cell death. Data are expressed as means of triplicate cultures. Percentage viability was determined by trypan blue exclusion. Quantitation of cells with apoptotic morphology yielded similar results. (A) Hydrogen peroxidase at 0.25 mM. Hydrogen peroxidase diluted 1:100 in phosphate-buffered saline was added to cell medium (5 x 10^5 cells per ml) at the beginning of the experiment. (B) Hydrogen peroxidase at 0.5 mM. (C) Hydrogen peroxidase at 1 mM.

Studies with temperature-sensitive mutants to define the biological effects of ABL oncogenes have shown that the effect on apoptosis is an early event in cell transformation by these oncogenes, allowing time for a later oncogene event (37, 38).

The discovery that Bcl-2 is essential in BCR-ABL transformation may also have implications for the therapy of BCR-ABL-related human leukemias, reinforcing the importance of the therapeutic modulation of apoptosis in the treatment of human cancer.

**BCR-ABL and the Pathogenesis of Ph+ Leukemia.** Acute leukemia derives from the clonal expansion of hematopoietic precursors that have lost their capacity to proceed to terminal differentiation. Leukemogenesis would therefore require accumulation of the minimum number of genetic events that result in accelerated cell growth and differentiation block. A large number of genetic alterations, including specific chromosome translocations, have been identified and causally linked to leukemogenesis, but the molecular basis of the composite leukemia phenotype remains largely unknown (39).

The property of BCR-ABL oncogenes of enhancing cell survival of Ba/F3 cells provides a cellular mechanism to explain their oncogenic action during Ph+ leukemogenesis. The demonstration that Bcl-2 is essential for transformation by BCR-ABL oncogenes, together with the fact mentioned above that these oncogenes also require MYC function (22), forces us to consider how the actions of these two genes might be connected. BCL2 has been found to cooperate with MYC in tumor induction or progression (31) and to antagonize MYC-induced apoptosis (40, 41). Therefore, the removal or blockage of BCR-ABL function might expose the cells to the apoptosis-inducing effects of MYC. Thus, one would expect that if the

**Fig. 4.** Suppression of mediated Bcl-2 RNA transcripts in BCR-ABL-expressing Ba/F3 cells transfected with pAbcl2. (A) Total RNA was isolated from Ba/F3+p190 (lane 1) and Ba/F3+p210 (lane 2) cells transfected with a vector expressing antisense mouse Bcl-2 (pAbcl2). Cellular RNA was hybridized to a human BCL2 cDNA probe. Autoradiography was for 9 h at −70°C. (B) Northern filter hybridization analysis of transfected (lanes 1 and 3) and untransfected (lanes 2 and 4) BCR-ABL-expressing Ba/F3 cells. An antisense mouse Bcl-2 oligonucleotide was used as a probe. The antisense Bcl-2 oligonucleotide used as a probe comprises the first 34 bases of the coding sequence of mouse Bcl-2 cDNA. Autoradiography was for 24 h at −70°C.

**Fig. 5.** Viability of BCR-ABL-expressing cells transfected with pAbcl2 after IL-3 deprivation. Duplicate cultures of cells (5 x 10^5 cells per ml) were cultured in medium lacking IL-3, and living cells were counted at daily intervals. All values are normalized to the relative viability of the initial number of cells.
expression of BCR-ABL\textsuperscript{100} is inhibited, apoptosis would reappear in these cells, and this has been observed (34). In contrast, when BCR-ABL is functional, this apoptotic pathway is blocked through Bcl-2 and the cells respond only to the proliferative function of MYC. Since MYC function is required for BCR-ABL-induced transformation (22), it is not unreasonable to suggest that BCR-ABL blocks MYC-induced apoptosis through Bcl-2. Indeed, recent studies have shown that cells harboring constitutively expressed forms of MYC are actually selected against, since inappropriately MYC expression activates the apoptotic program in the affected cell (42, 43). However, if the cell also contained an activating mutation in a gene that blocks apoptosis, the full transforming potential of a deregulated MYC gene would become evident. To date, the only other example of this type of cooperation with MYC is BCL2 itself (40, 41).

Oncoproteins that inhibit cell death (BCR-ABL, BCL2) cooperate with those that induce proliferation but cannot overcome death (MYC), which implies that cancer results from the combination of induction of proliferation and escape from death.

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